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Fifteenth day of July 2004

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PROVISIONAL SPECIFICATION

Applicant:

THE UNIVERSITY OF QUEENSLAND

Invention Title:

PRODUCTION OF HYDROGEN

The invention is described in the following statement:

PRODUCTION OF HYDROGEN

Technical Field

The present invention is concerned with the production of hydrogen and, more particularly, with the enhancement of hydrogen production in photosynthetic microorganisms with natural hydrogen production capability. In particular the focus is on photosynthetic microorganisms, including cyanobacteria and algae that are able to produce hydrogen from water. The invention also relates to the manipulation of the physiology of such organisms in order to enhance hydrogen production.

Background Art

The development of a clean, sustainable and economically viable energy supply for the future is one of the most urgent challenges of our generation. Oil production is expected to peak in around 5 to 30 years time and economically viable oil reserves will be largely depleted by 2050. As a result, there is a need to develop a clean, sustainable and economically viable supply for the future. As such, there is now a concertad international effort to switch from a fossil fuel economy to a hydrogen economy. However, a viable hydrogen economy requires clean, sustainable and economic ways of generating hydrogen. Current hydrogen production depends almost entirely on the use of non-renewable resources (i.e. steam, reformation of natural gas, coal gasification and nuclear power driven electrolysis of water). Although these approaches are initially likely to drive a transition towards a hydrogen economy, the hydrogen produced is more expensive per unit energy than the non-renewable energy source from which it is derived. In addition, the use of fossil fuels and nuclear power is unsustainable. Therefore, there is a clear need to establish economically viable means of hydrogen production.

A particularly desirable source is the production of hydrogen using photosynthetic organisms, since the

ultimate energy source is solar energy. Higher plants, green algae and cyanobacteria are all highly evolved in their ability to capture solar energy, with photon conversion efficiencies as much as 80%. Solar energy is captured and stored in the form of starch and other molecules including proteins, which are subsequently used as a fuel to drive ATP production via the processes of oxidative phosphorylation in the mitochondria (Fig.1) This H+ and electron store, shown here as starch can be channelled to generate H_2 in a highly efficient manner using algal bioreactors.

In the first step of photosynthesis, PSII drives the most highly oxidizing reaction known to occur in biology, splitting H_2O into O_2 , H^+ and e^- (Fig.1). O_2 is released into the atmosphere and is responsible for maintaining aerobic life on Earth. The derived e^- are passed along the photosynthetic e^- transport chain (Fig.2) from PSII via plastoquinone (PQ) to cytochrome b_6f (cyt b_6f) and Photosystem I (PSI) and are ultimately used in the production of NADPH. In a parallel process (photophosphorylation), H^+ are released into the thylakoid lumen (Fig.1) where they generate a H^+ gradient that is used to drive ATP production via ATP synthase. NADPH and ATP are subsequently used to produce starch and other biomolecules.

ATP and NADPH/NADH are fundamental requirements of all living cells. The inhibition of PSII (eg. incubation in the dark) blocks the supply of H^+ and e^- that are used to generate ATP and NADPH via photophosphorylation in the chloroplast. For a time, the shortage of ATP and NADPH caused by the inhibition of PSII can be compensated for via aerobic respiration mediated by the mitochondrial e^- transport chain (Fig.1), which metabolises starch, proteins and lipids. As its name suggests, oxidative phosphorylation requires O_2. The O_2 is combined with H^+ and e^- by mitochondrial Complex IV to generate H_2O, which essentially acts as an e^- sink (Fig.1).

Under anaerobic conditions (Fig.2) Complex IV is inhibited, blocking e^- transport through the remainder of the e^- transport chain consisting of Complexes I, II, III and Cytochrome C (Cyt C). Under strictly anaerobic conditions most photosynthetic organisms die. However, a select number of photosynthetic organisms such as the green alga *C. reinhardtii* have a third mechanism, which allows them to switch into a mode of ATP and NADPH production (Fig.2). Under illuminated anaerobic conditions, they generate ATP in the chloroplast while simultaneously producing H_2 (Fig.2-CHL HydA) as a volatile e^- sink, instead of H_2O (Fig.2-MIT Complex IV). This process involves the Hydrogenase HydA located in the chloroplast stroma (Florin et al, 2001 and Happe and Kaminski, 2002.). HydA transcription and activity is strongly inhibited by O_2 (Ghirardi et al, 1997). This inhibition may have evolved to ensure that, when possible, ATP is produced using the more efficient pathway of mitochondrial oxidative phosphorylation.

The earliest reports of algal H_2 production date back to the 1930s (Stephenson and Stickland, 1931). It was discovered that certain green algae and cyanobacteria could produce H_2 gas upon illumination, by a reaction that was extremely sensitive to inhibition by O_2. Despite the obvious attraction of using photosynthetic organisms for sustainable H_2 production from H_2O it was not until 2000, that Melis and co-workers first reported a method to overcome this inhibition. (Melis, 2000 and US Patent Application No. 2001/005343 Melis) describes a process in which the inhibition was lifted by temporally separating the O_2 generating H_2O splitting reaction, catalysed by PSII, from O_2 sensitive H_2 production catalysed by the chloroplast Hydrogenase (HydA). This separation was achieved by culturing *C. reinhardtii* in the presence and absence of Sulphur. Sulphur is required for the *de novo* synthesis of the D1 protein of the PSII reaction centre. The D1 protein has an approximate half life of 30min,

being damaged under non-optimal conditions through the highly oxidizing reaction that it drives. In the presence of sulphur, high levels of active PSII are maintained and H_2O is split into H^+, e^- and O_2. The H^+ and e^- are subsequently recombined by HydA to generate H_2 under Sulphur depleted conditions, which lift the inhibition of the Hydrogenase induced by O_2. This for the first time, facilitated long-term H_2 production using wild type (wt.) *C. reinhartii*.

The Melis process is, however, subject to considerable practical difficulties. The actual rate of hydrogen gas accumulation is at best 15 to 20% of the photosynthetic capacity of the cells (Melis and Happe 2001) and suffers the inherent limitation that hydrogen production by S deprivation of the algae cannot be continued indefinitely. The yield begins to level off after about 70 hours of S deprivation, and after about 100 hours of S deprivation the algae need to go back to normal photosynthesis to be rejuvenated by replenishing endogenous substrates. Consequently there remains a need to identify a sustainable process of photosynthetic hydrogen production.

Summary of the Invention

The present invention is based upon the surprising observation that disruption of the regulation of the oxidative phosphorylation pathway in the mitochondria affects the chloroplast in a fashion which increases ATPase levels in the chloroplast and reduces oxygen production by PSII but permits electron transfer to PSI, and therefore allows operation of a hydrogenase (HydA) that accepts electrons from PSI, with a reduction in the inherent oxygen inhibition.

Accordingly, a first aspect of the present invention provides a process for the production of hydrogen, comprising the steps of:

(1) providing a photosynthetic organism having electron transfer capability through a photosynthetic

"light" reaction involving photosystems I and II (PSI and II) and an oxidative phosphorylation pathway, said organism being capable of expressing a hydrogenase in the chloroplast in order to produce hydrogen when illuminated in the absence of oxygen, characterised in that electron transfer along the oxidative phosphorylation pathway is diverted, at least in part, to the photosynthetic electron transport chain in the chloroplast downstream of PSII;

(2) growing a culture of the microorganism under illuminated conditions on a medium suitable for expression of the hydrogenase;

(3) sealing the culture from atmospheric oxygen; and

(4) collecting the evolved hydrogen.

A number of organisms are physiologically suited to use in this process. In particular, photosynthetic microorganisms capable of using water as an indirect substrate for hydrogen production and which include a hydrogenase are used, and these include cyanobacteria and algae. Preferably the organisms are selected from *Chlamydomonas reinhardtii*, Scenedesmus obliquus, Chlorococcum littorale and Chlorella fusca. The alga *Chlamydomonas reinhardtii* is particularly preferred as processes for the genetic manipulation of the organism are well developed and its genome has been sequenced. A high hydrogen producing mutant (*stm6*) is used in a particularly preferred embodiment of the present invention. *Stm6* has a reduced level of the mitochrondrial enzyme Cytochrome C oxidase, and it is therefore preferred that the photosynthetic organism have reduced Cytochrome oxidase activity. While not wishing to be bound by theory, it is believed that the lack of Cytochrome C oxidase disrupts the oxidative phosphorylation pathway, diverting at least some electrons to the photosynthetic electron transport chain in the chloroplast via plastoquinone. As a result that plastoquinone remains in a relatively reduced state (PQH2) even without electrons supplied by PSII as the

oxidised PQ rather than the reduced PQH2 accepts electrons from PSII the rate of electron transport through PSII is reduced resulting in reduced O2 production rates. The lower oxygen production appears to result in less inhibition of the hydrogenase, hydA, in *C. reinhardtii*.

Any suitable means of disrupting oxidative phosphorylation may be employed. In a preferred embodiment of the invention relative expression of Cytochrome C oxidase is reduced. In particular, the transcription of *Cox I* may be reduced through a reduction in the expression of the regulatory gene, Moc I. *Stm6* is a Moc I knock-out and has been shown to produce hydrogen at the rate 15 to 16 times that of the wild type under the conditions tested.

The rate of hydrogen production may be further increased by uncoupling PSII from PSI. Uncoupling of the photosystems may be achieved by any suitable means, such as through the addition of a chemical uncoupler such as CCCP.

According to a second aspect of the present invention there is provided an isolated cell capable of photosynthesis which also has hydrogen production capability through expression of a hydrogenase, said cell having electron transfer capability through a photosynthetic "light" reaction involving PSI and PSII and an oxidative phosphorylation pathway, characterised in that electron transfer along the oxidative phosphorylation pathway is diverted, at least in part, to the PSII/PSI transition.

In the organism of the invention, plastoquinone is substantially in a reduced state.

Typically the diversion of electrons from the oxidative phosphorylation pathway is due to a reduction in Cytochrome C oxidase activity, as described above, hence the cell has reduced Cytochrome C oxidase in the mitochondria, and also reduced ATP synthase concentration, lower cellular ATP and lower cellular oxygen

concentrations. Advantageously the cell is a Moc I knockout.

The cell may be a photosynthetic cell from a higher organism, but is preferably an alga, most preferably *Chlamydomonas reinhardtii*, and typically a Moc I knockout. A mutant strain of *Chlamydomonas reinhardtii* referred to herein as such as *stm6* was deposited with the Culture Collection of Algae and Protozoa (CCAP) on 1 July 2003 under CCAP accession number 11/129.

Methods for cultivation of suitable microorganisms and methods for collection of evolved hydrogen are known to the person skilled in the art and are described, for example, in US Patent Application No. 2001/005343 and by Melis (Melis, 2000), the contents of which are incorporated herein by reference.

Brief Description of the Drawings

Figure 1 is a diagrammatic representation of photosynthesis, carbohydrate metabolism and oxidative phosphorylation as it operates under aerobic conditions;

Figure 2 shows photosynthetic hydrogen production in *C. reinhardtii* under anaerobic conditions;

Figure 3 is a graph showing H_2 production properties of wild type (wt) and mutant (M) *C. reinhardtii*. *A)* Under anaerobic conditions the mutant produced approximately 6x more H_2 than the wt, over a range of light intensities. B) Under anaerobic conditions and in the presence of the uncoupler CCCP, the mutant produced approximately 15x more H_2 than the wt, over a range of light intensities. This uncoupler facilitates the free H^+ flow across the thylakoid membrane. The H^+ that migrate to the stroma through the action of CCCP appear to act as substrate to HydA;

Figure 4 shows electron micrographs of stained sections of Wt and high H_2 producing mutant *C. rheinhardtii*. C-chloroplast, G- Golgi, M-Mitochondria, N-Nucleus, S-Starch. Note that: 1) the grana of the mutant

are much less stacked than those of the wt. This has implications for the light capturing properties of the mutant. The mutant has large carbohydrate stores in the form of starch. The H^+ and e^- derived from water are stored in starch, before being converted to H_2 by HydA as described in Fig.2.

Figure 5 provides an analysis of light-induced short-term adaptation mechanisms in the chloroplast:

a) State transitions analysed by 77k fluorescence spectroscopy in *WT*, *B13* and *stm6*

b) Fluorescence video image of *WT*, *B13* and *stm6* on TAP-agar plates in state 2 and state 1 (Illumination with actinic light at 620nm ± 15min illumination with 710nm PSI light)

c) *In vivo* phosphorylation protein pattern of thylakoid membranes isolated from state2-adapted *WT* and *stm6* cell

Figure 6 shows that *stm6* is sensitive to light stress:

a) *Wt*, *B13* and *stm6* strains, cultivated to their mid logarithmic growth phase in TAP medium, exposed to 1000µmol m^{-2} s^{-1} white light for 4 hours, subsequently inoculated on TAP agar plates and grown for 7 days in 40µmol $m^{-2}s^{-1}$ white light.

b) High light cell rates surviving high light treatment over a period of 4 hours in *WT*, *B13* and *stm6* strains. Cells were diluted to $1x10^4$ cells/ml prior to light treatment;

Figure 7 illustrates the genomic DNA site in *stm6* affected by the tandem nuclear insertion of two pArg7.8 plasmids:

- by sequence analysis after chromosome walking LMS-PCR) and:
- by PCR analysis with specific moc1, toc1 and pArg7.8 primers.

The 2 black arrow sets mark the primer pairs used for PCR

The 2 grey arrows mark the integration sites of the nuclear insertion in *Wt*

Figure 8 gives the protein sequence of MOC1 and alignment with the human transcription termination factor mTERF. The model and the boxes mark the locations of the mitochondrial transit sequences and the identified mTERF domain structures in MOC1.

Figure 9 is a western analysis and ATP level measurements in *WT*, *stm6* and *B13*:

a) immunoblot of light grown cells using antibodies specific for cytochrome oxidase subunit COX90

b) immunoblot using anti-peptide antibodies to demonstrate localisation of MOC1 to the mitochondrion and not the chloroplasts (anti-D2 and anti-Cyt c blots were performed as controls for chloroplasts and mitochondria, respectively) and to demonstrate up-regulation of AOX1 in *stm6*. Relative intensities of cross-reactions, as determined by densitometry, are indicated.

c) ATP levels in cells of *WT*, *stm6* and *B13* grown in the light and dark.

Figure 10 shows evidence for the role of MOC1 in light-induced mitochondrial transcription regulation:

a) Semi-quantitative RT-PCR of *nad2* and *cox1* in dark-grown cultures and after exposure to light (3hours 200µmol $m^{-2}s^{-1}$); mRNA levels were normalised to that of actin.

b) moc1-Northern Blots of isolated total RNA from dark- and light-grown *WT*

c) anti-MOC1 and anti-cytochrome c immunoblots of mitochondria isolated from *WT* cells exposed to high light (800µmol m^{-2} s^{-1}) for 0 and 180min. The *stm6* blot was performed as a control.

d) Cytochrome c oxidase activity measurements in isolated mitochondria from *WT* and *stm6* (3hours 200µmol $m^{-2}s^{-1}$)

Figure 11 is an illustration of a model

describing the role of MOC1 in the regulation of mitochondrial respiratory electron transport upon high light stress and its influence on light-induced redox control processes in the chloroplast. Grey boxes represent organelles.

Detailed Description of the Invention

Preferred embodiments of the present invention will now be described. In seeking to identify cellular components important for the redox-controlled regulation of photosynthesis the approach was to screen for mutants that are perturbed in state transitions. This is a redox-controlled mechanism that balances the excitation of the two photosynthetic reaction centres involved in linear electron flow, photosystems I and II (Bonaventura and Myers, 1969; Murata, 1969). When the plastoquinone pool, which is a component of the photosynthetic electron transport chain linking PSII and PSI, becomes more reduced, mobile light-harvesting antennae detach from PSII, following redox-activated phosphorylation, and dock with PSI thus increasing the excitation of PSI and driving the PQ pool more oxidised (into so-called state 2). The reverse process occurs when the PQ becomes more oxidised (to achieve state 1) (Allen *et al.*, 1981; Horton *et al.*, 1981). A rapid chlorophyll-fluorescence based screen was employed to identify colonies of the model organism, the green alga *Chlamydomonas reinhardtii*, that are blocked in state transitions (the inter conversion of state 1 and state 2) (Kruse *et al.*, 1999; Fleichmann *et al.*, 1999). A gene that is important for state transitions was identified. Surprisingly the gene product was found to be a nuclear encoded protein targeted to the mitochondrion not the chloroplast and is involved in regulating mitochondrial gene expression.

Example 1 - Isolation of Mutant Strains

Strains and culture conditions

The *Chlamydomonas reinhardtii* strains *Wt13* and *CC*

1618 (arg7 cw15, mt⁻) were obtained from the *Chlamydomonas* Genetics Center Collection (Duke University, USA). All strains used were cultivated mixotrophically in TAP medium (Tris-acetate-phosphate, pH 7.0) by illumination with 40 μmol m^{-2} sec^{-1} white light at 20 °C (Harris 1989) in a twelve hours light-dark cycle to a cell density of $2x10^6$ cells per ml.

When required (for the arginine auxotroph strain *CC1618*) the medium was supplemented with 110μg arginine per ml.

Mutant construction and genetic analysis

Nuclear transformation was performed as described following the methods of (Kindle *et al.*, 1989) and (Purton and Rochaix, 1995).

Plasmids pARG7.8, containing a 7.8 kb genomic DNA fragment of the *Chlamydomonas reinhardtii* argininosuccinate lyase gene (Debuchy et al. 1989) and a 0.4 kb fragment of bacteriophage ϕX174 DNA (Gumpel and Purton 1994), were used for transformation experiments. Prior to use pARG7.8 was linearised by digestion with BamHI.

Genetic crosses were performed with *Wt13* and the mutant as described by Harris (1989).

DNA sequences flanking the tag were cloned by ligation mediated suppression PCR (LMS-PCR) (Strauss *et al.*, 2001) and by plasmid rescue (Tam and Lefebvre, 1993).

Isolation of state transition mutants

To identify genes involved in state transitions within *C. reinhardtii*, we screened a library of mutants that can grow on TAP medium lacking arginine, generated after the random insertion of plasmid pArg7.8 carrying the Arg7 gene into the nuclear genome of the arginine auxotrophic strain, CC1618 (Debuchy *et al.*, 1989; Gumpel and Purton, 1994). A plate-based fluorescence video-imaging screen, which involves the recording of chlorophyll fluorescence from individual colonies before and after illumination with light that induces state

transitions in *Wt* was used to identify potential state transition mutants (*stm*) (Kruse et al., 1999). Of the 2 x 10^4 colonies screened, four possible *stm* mutants were identified, with the mutant *stm6* being demonstrated to have included hydrogen production.

Fluorescence video imaging and 77K fluorescence spectroscopy

Screening of the mutants for state transitions defects was performed as described earlier (Kruse et al., 1999) by video imaging with a FluorCam 700 MF apparatus (Photon System Instruments) at room temperature and at 77K with a fluorescence/luminescence spectrometer (Perkin Elmer LS50B). Samples were measured following illumination with 40µmol m^{-2} s^{-1} white light (state 2) or illumination for 20min with 710nm (Schott filter, Germany) PSI light (state 1). Alternatively, cells were adjusted to state 2 by incubation in the dark for 30min under nitrogen atmosphere according to Bulté et al. (1990).

Example 2 Characterisation of stm6 Mutant

stm6 is blocked in state 1 and impaired in PSII-LHCII phosphorylation

Chlorophyll fluorescence assays conducted by fluorescence emission spectroscopy at 77K (Figure 5) and chlorophyll video imaging with actinic 620nm light at room temperature (Figure 5) revealed that *stm6* was blocked in state 1. In contrast to the *WT*, light preferentially absorbed by PSII was unable to drive the cells into state 2, which is monitored by an increase in the fluorescence coming from PSI at 720nm (Figure 5). A transition to state 2 were also blocked when the PQ pool was driven reduced in the dark through anaerbiosis (data not shown).

Since state 2 is associated with the phosphorylation of threonine residues in the light harvesting (LHC) antenna proteins of PSII (Allen et al., 1981), anti-phosphothreonine antibodies were used to

assess the level of phosphorylation in immunoblots. *stm6* showed a marked reduction in the phosphorylation levels of CP29 (P9) and LHC-P11 proteins upon illumination conditions that promote phosphorylation of these proteins in *WT* (Figure 5). Control immunoblots confirmed that *stm6* still contained WT levels of LHC proteins (Figure 5).

To test that the PQ pool could still be reduced in *stm6*, chlorophyll fluorescence measurements were performed to determine the photochemical quenching parameter, q_p, which is a measure of the reduction state of the PQ pool (Lunde et al., 2000). Under growth light conditions the PQ pool was in a more reduced state in *stm6* compared with *WT* ($1-q_p$ of 0.77 in *stm6* and 0.59 in *WT*) (Table 1). The levels of PSI and PSII activity in *stm6* were also similar to that found in WT (Table 1).

stm6 is sensitive to high light

Besides effects on state transitions, a striking phenotype of the *stm6* mutant was its sensitivity to light stress. After 4 hours illumination by 1000 µmol m^{-2} s^{-1} high light cells of the mutant showed a marked decline in viability whereas the *WT* and a complemented strain, *B13*, were nearly unaffected (Figure 6). *stm6* showed a dramatic increase in singlet oxygen inside the cell upon illumination as assessed by the accumulation of lipid hydroperoxides (Table 1).

Disruption of a single mterf-type gene in stm6 is responsible for the defect in state transitions and for light sensitivity

To test whether the *arg* marker carried by plasmid pArg7.8 was tightly linked to *stm6*, genetic crosses were performed between *Wt13* and *stm6* and the progeny scored for the ability to grow on arginine-free medium and to do state transitions as assessed by video imaging. In all 50 progeny examined, clones able to grow on arginine-free TAP medium exhibited normal state transitions whereas clones showing arginine auxotrophy also showed impaired state transitions (data not shown). These data suggested that

stm6 was an *arg*-tagged mutant.

A combination of plasmid rescue and ligation-mediated suppression (LMS)-PCR (Strauss et al., 2002) led to the identification of the site of insertion of the *arg* marker in *stm6*. More detailed analyses revealed that two pArg7.8 plasmids had inserted in tandem into *stm6* (Figure 7).

Subsequent sequence analysis and homology searches revealed that two genes were affected by the random integration of the pArg7.8 plasmid. One gene was previously described as a nuclear transposon (toc1) (Day *et al.*, 1988), the second gene was unknown and its sequence submitted to *GenBank* database as moc1 (*Genbank* AccNo. AF531421). The correct localisation of the affected genomic insertion site in *stm6* DNA was finally confirmed by sequencing and PCR analysis (Figure 7).

The random insertion by non-homologous recombination caused the deletion of approx. 2kb of genomic DNA including 610bps at the 3' region of the moc1 gene and 880bps at to the 5' of the toc1 gene (Figure 7).

PCR analysis in *stm6* and *Wt* with a 5'-specific moc1-primer and a second primer derived from the nuclear insertion sequence resulted in the amplification of a 1005 bp PCR product in *stm6*. This confirmed that the insertion caused the deletion of only parts of moc1 leaving the 5' region of the gene untouched. The identification of remaining 512bps of moc1 on the 5' region of the nuclear insertion and of remaining 4782bps of toc1 on the 3' region of the nuclear insertion in *stm6* clearly demonstrated that the mutation is entirely restricted to the 3'-moc1/5'-toc1 gene region and did not affect any other possible adjacent coding regions.

Complementation experiments confirmed that the single copy of moc1 was responsible for the lack of state transitions in *stm6* (see Figures 5, 6, 9). The complemented strain *B13* was isolated in a co-transformation approach using a moc1-containing cosmid in

combination with a second vector containing the cry1 gene conferring emetine resistance as a dominant selectable marker (Nelson *et al.*, 1994). The 37kb cosmid was isolated from a cosmid library (kind gift of Dr. Saul Purton, UCL, UK). Sequence analysis of the insertion region revealed that moc1 was the only gene inserted into the cosmid.

Of approximately 5000 emitine-resistant colonies assessed, four were found to perform normal state transitions (assessed by fluorescence video imaging) and all four contained the moc1 gene.

The moc1 gene encodes a 35kDa protein containing somewhat surprisingly a putative mitochondrial transit sequence and two mitochondrial transcription termination factor domains (mterf) with leucine zipper motifs characteristic of DNA-binding proteins (Daga *et al.*, 1993). Overall there were striking similarities to the 35kDa human mTERF protein (34% based on alignments covering the full length sequences) (Figure 8) and their homologues in *Drosophila melanogaster* (DmTTF, Roberti *et al.*, 2003) and *sea urchin* (mtDBP, Loguercio *et al.*, 1999). Human mTERF binds downstream of tRNA genes and is thought to be involved in controlling the amount of tRNA and rRNA synthesised within the mitochondrion as well as the expression of other mitochondrial genes and consequently the functionality of the mitochondrial respiratory chain (Fernandez-Silva *et al.*, 1997; Selwood, 2000; Hess *et al.*, 1991). Interestingly 9 homologues to MOC1 with mterf domains have been identified in the genome of *Arabidopsis thaliana*, four of which are predicted to be targeted to the mitochondrion (At1g61980, At2g44020, At4g02990, At2g03050) and one to the chloroplast (At5g55580). Analysis of the recently released *Chlamydomonas* nuclear genome data-base, plus DNA hybridisation experiments, suggest that there is only one copy of moc1 in *C. reinhardtii*.

MOC1 is a mitochondrial protein and its absence affects the expression of cytochrome oxidase

Anti-peptide antibodies raised against MOC1 were used to confirm that MOC1 was indeed targeted to the mitochondrion rather than the chloroplast (Figure 9) and that it was absent in the *stm6* mutant. Importantly expression of MOC1 was up-regulated at both the RNA and protein level upon a dark to light transition (Figure 10).

Experiments were performed to assess whether the absence of MOC1 had affected the expression of mitochondrial respiratory complexes. Immunoblots revealed that accumulation of subunit 90 of cytochrome oxidase (Lown *et al.*, 2001) (COX90) was reduced in *stm6* (Figure 9) whereas levels of soluble cytochrome c were unaffected and levels of the alternative oxidase 1 (AOX1) even increased by the mutation (Figure 9). In the complemented strain, *B13*, subunit 90 of cytochrome oxidase were restored to almost *WT* levels. The immunoblot data agreed well with activity measurements for cytochrome oxidase in mitochondria isolated from dark-grown *WT* and *stm6* cells (Figure 10). Upon light treatment, there was an increase in activity in *WT* but not *stm6*. Levels of ATP in light-grown *stm6* cells were at about 50% of the levels of the *WT* and *B13* (Figure 10), consistent with a role for cytochrome oxidase in the generation of ATP from photosynthetically derived reductant, in line with earlier suggestions (Krämer and Heldt, 1991).

MOC1 is involved in changing the transcription of the mitochondrial genome upon exposure to light

Given that MOC1 encodes a transcription termination factor homologous to human mTERF, experiments were directed at detecting possible perturbations to the transcript profile of the mitochondrial genome, which for *C. reinhardtii* consists of a 15.8 kb linear genome (Gray and Boer, 1988). A number of respiratory subunits are encoded by the genome including subunit 1 of cytochrome oxidase (*cox1* gene product), 5 subunits of complex I (*nad1-2, nad4-6* gene products), and apocytochrome b (*cob* gene product).

Semi-quantitative RT-PCR analysis of the *nad2* and *cox1* genes revealed dramatic differences in transcript levels between *WT* and *stm6* following exposure of dark-grown cells to light. Compared to the *WT*, *stm6* consistently showed reduced levels of the *cox1* transcript and higher levels of the transcript derived from the *nad2* gene, located immediately downstream of *cox1* (Figure 10).

Together these results suggest an important role for MOC1 in controlling the level of cytochrome oxidase activity at the level of transcription of *cox1*. Since COX1 is the first subunit to be assembled into the cytochrome oxidase complex, the level of COX1 expression is likely to be an important determinant of the amount of enzyme synthesised (Nijtmans et *al.*, 1998).

A model for the role of MOC1 in the regulation of the mitochondrial respiratory chain during light stress

A model for the role of MOC1 in mitochondrial function is presented in Figure 11. The mitochondrial genome is transcribed bi-directionally to give two primary transcripts, which are then processed into smaller transcripts (Gray and Boer, 1988). Our results show that MOC1 is important for maintaining a high level of *cox1* mRNA with respect to the downstream *nad2* transcript. A possible binding site for MOC1, which is likely to be a transcription termination factor, would therefore be between *cox1* and *nad2*. The diversity of binding sites for this class of transcription factors make it difficult at this stage to predict a possible binding site for MOC1 (Roberti et al., 2003).

In our model the activity of MOC1 would allow the mitochondrion to disconnect the transcription of *coxI* from the downstream *nad* genes so as to increase the relative expression of COX1 needed for the synthesis of additional cytochrome oxidase complexes. This is particularly relevant given that rotenone-insensitive mitochondrial NADH dehydrogenases, unrelated to complex I, might be induced in the light (Svensson and Rasmussen, 2001) so

that there would be a need for the synthesis of more cytochrome oxidase. Given that there might be more than one binding site for MOC1 per genome (Roberti *et al.*, 2003) and there is potential for binding to RNA as well as DNA, the effects of MOC1 activity on mitochondrial gene expression might be rather complex.

Table 1

	WT	*Stm6*	*B13*
ϕ PSI activity (thylakoids)	set to100	130±10	95±10
ϕ PSII activity (thylakoids)	set to100	75±15	105±10
Chl a/b ratio (cells)	1,95±0,1	1,85±0,1	1,9±0,1
MDA concentration	set to 100	230±20	110±15
1-qP (Fm'-Fs)/(Fm'-Fo')	0,59±0,02	0,77±0,02	0,60±0,02

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The disclosure of the following documents is incorporated herein through reference:

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Dated this 7th day of July 2003

THE UNIVERSITY OF QUEENSLAND

By their Patent Attorneys

GRIFFITH HACK

Fellows Institute of Patent and

Trade Mark Attorneys of Australia

Figure 1

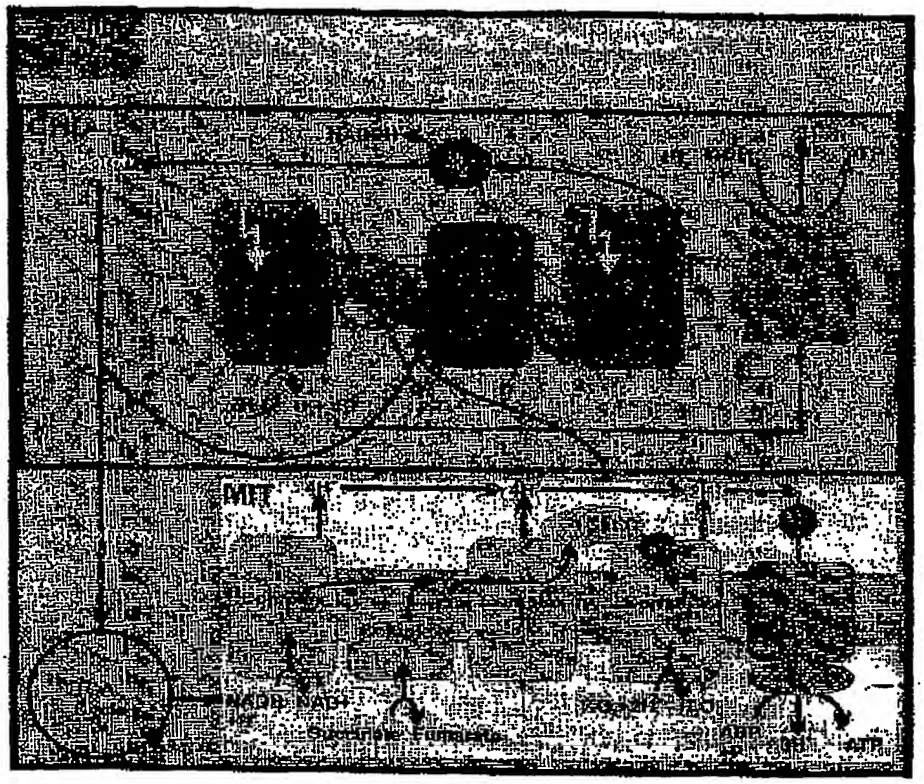
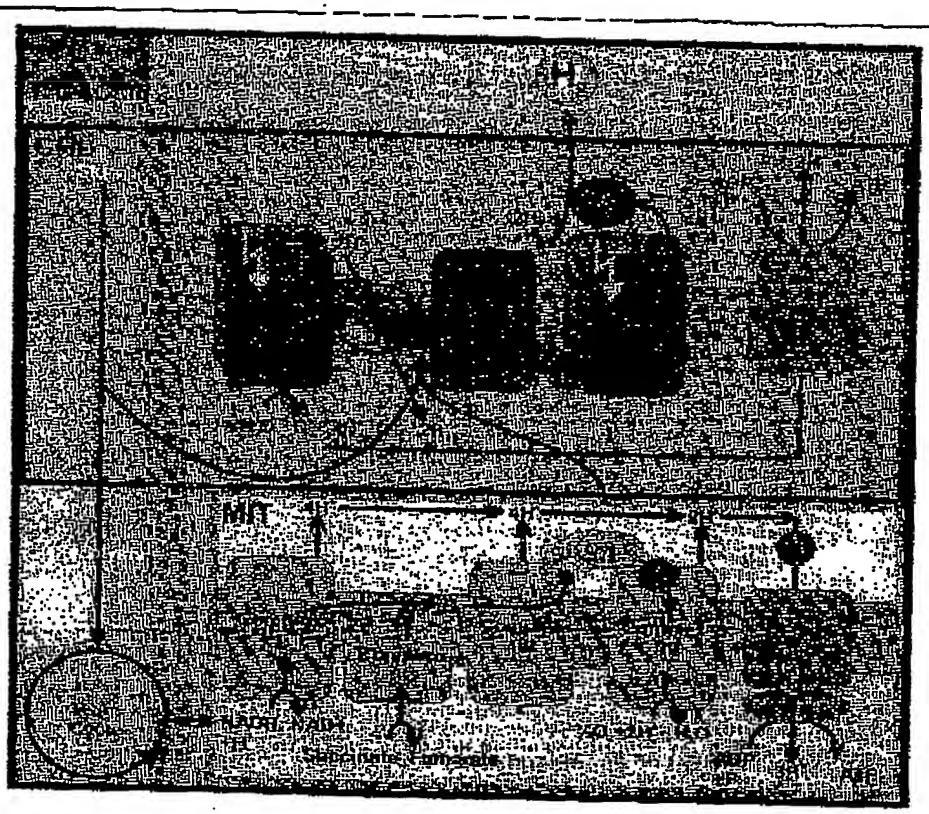


Figure 2



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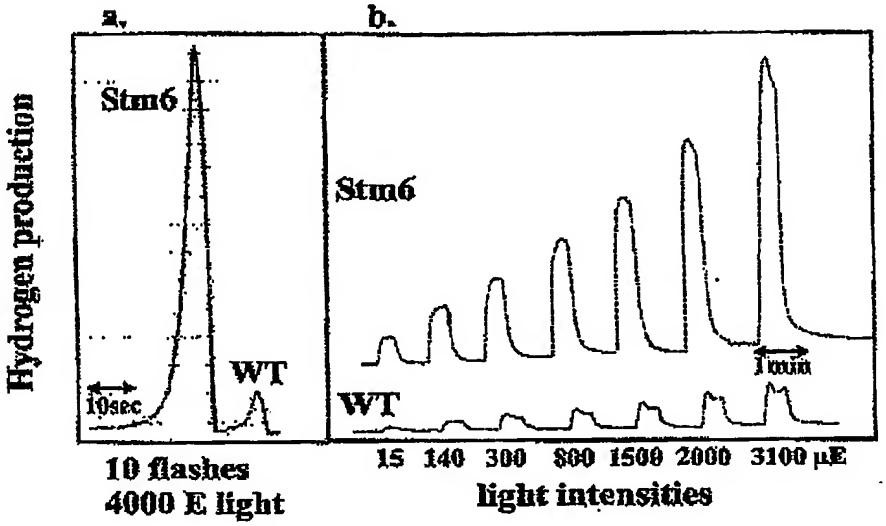

a.
b.
Hydrogen production
Stm6
WT
10sec
10 flashes
4000 E light
Stm6
WT
1min
15 140 300 800 1500 2000 3100 µE
light intensities

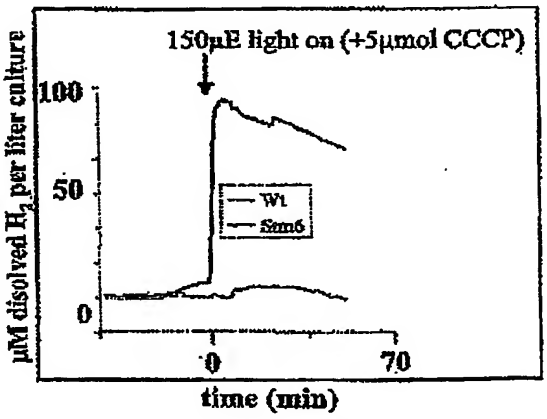

150µE light on (+5µmol CCCP)
µM disolved H2 per liter culture
100
50
0
Wt
Stm6
0
70
time (min)

Figure 4

Figure 5

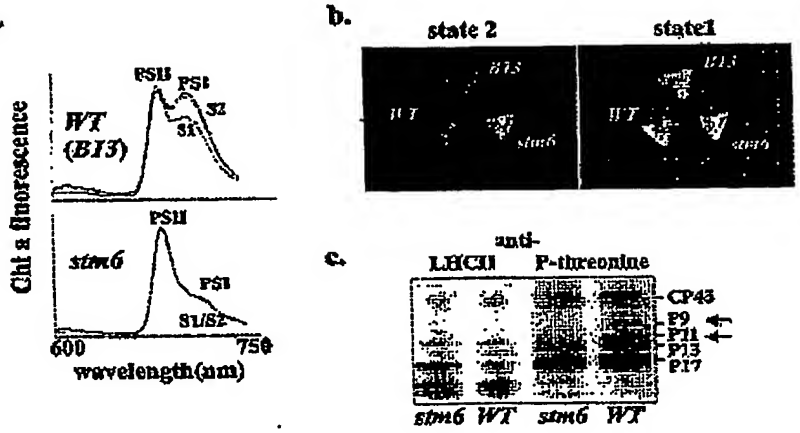

a.
PSII
PSI
S2
S1
WT
(B13)
Chl a fluorescence
PSII
stm6
PSI
S1/S2
600
750
wavelength(nm)
b.
state 2
state1
B13
WT
stm6
c.
anti-
LHCII
P-threonine
CP43
P9
P11
P13
P17
stm6 WT stm6 WT

Figure 6

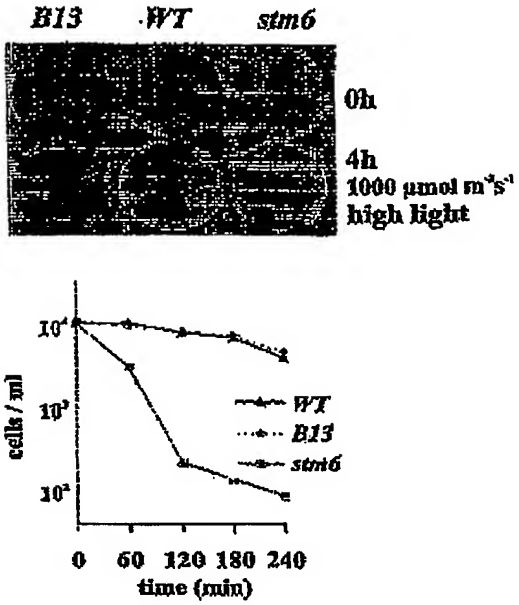

B13
WT
stm6
0h
4h
1000 µmol m⁻²s⁻¹
high light
cells / ml
10⁴
10³
10²
WT
B13
stm6
0
60
120
180
240
time (min)

Figure 7

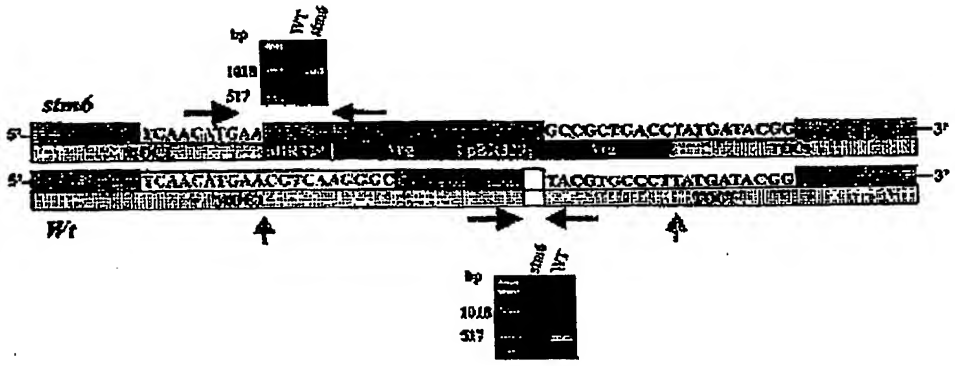


Figure 8

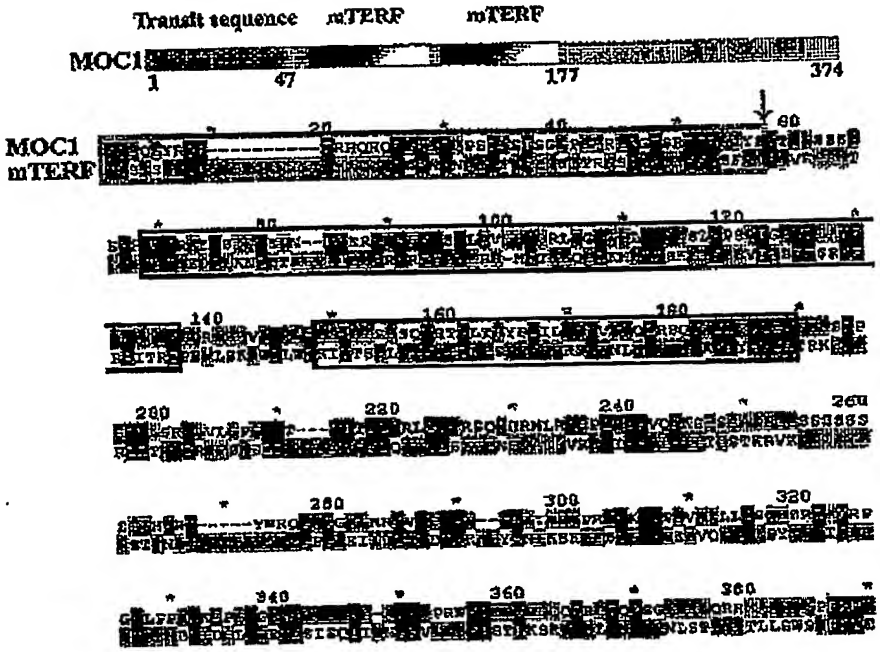


Figure 9

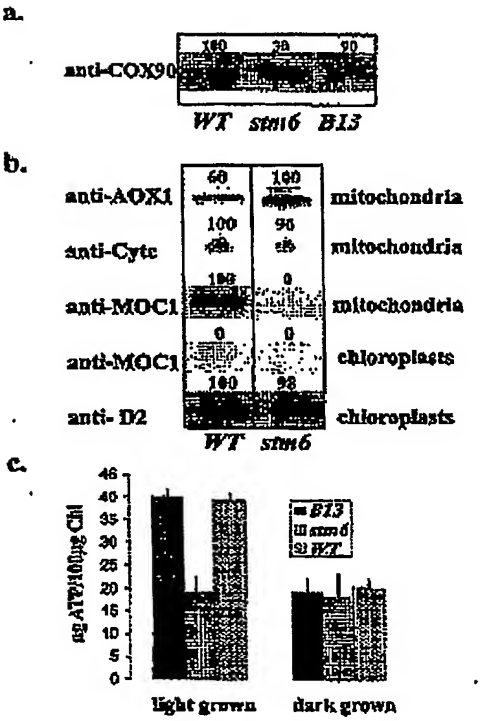
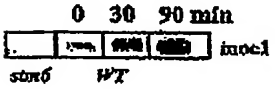

a.
anti-COX90
WT stm6 B13
b.
anti-AOX1
anti-Cytc
anti-MOC1
anti-MOC1
anti- D2
mitochondria
mitochondria
mitochondria
chloroplasts
chloroplasts
WT stm6
c.
ng ATP/100μg Chl
B13
stm6
WT
light grown
dark grown

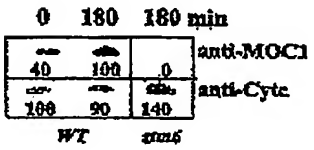
Figure 10

a.

b.



c.



d.

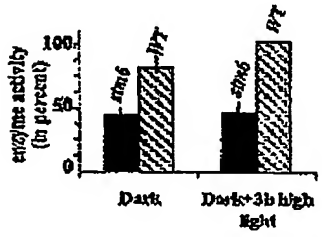
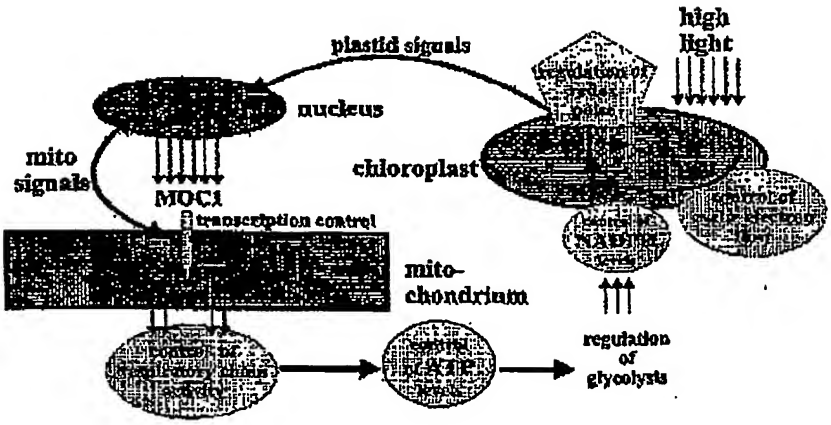


Figure 11


plastid signals
high light
nucleus
mito signals
chloroplast
MOC1
transcription control
mito-chondrium
regulation of glycolysis